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AMPK breathing and oxygen supply

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Abstract

Regulation of breathing is critical to our capacity to accommodate deficits in oxygen availability and demand during, for example, sleep and ascent to altitude. Key to this are two reflex responses, hypoxic pulmonary vasoconstriction (HPV), which aids ventilation-perfusion matching at the lungs, and the hypoxic ventilatory response (HVR) which accelerates ventilation. In 2004 I proposed that HPV might be mediated by the AMP-activated protein kinase, which governs cell autonomous metabolic homeostasis. Pharmacological evidence was presented in support of this view, and the hypothesis extended to incorporate a role for AMPK in regulating carotid body afferent input responses during hypoxia and thus the HVR. The present article reviews our subsequent findings on these matters and those of others, which provide strong support for the view that AMPK mediates HPV. AMPK is also critical to the HVR, but against our expectations it is not required for carotid body activation during hypoxia. Contrary to current consensus in this respect, our findings suggest that AMPK deficiency blocks the HVR at the level of the brainstem, even when afferent input responses from the carotid body are normal. We have therefore revised our hypothesis on the HVR, now proposing that AMPK integrates local hypoxic stress at defined loci within the brainstem respiratory network with an index of peripheral hypoxic status, namely afferent chemosensory inputs. Nevertheless, in general outcomes are consistent with the original hypothesis, that the role of AMPK has evolved, through natural selection, to extend to the regulation of breathing, and thus oxygen and energy (ATP) supply to the whole body.

Introduction

The mechanisms of hypoxia-response coupling remain keenly debated topics, nowhere more so than with respect to hypoxic pulmonary vasoconstriction (HPV) which aids ventilation-perfusion matching at the lungs, and the hypoxic ventilatory response (HVR) which increases breathing rates to compensate for reductions in O₂ supply. In

2001 I developed the hypothesis (Evans, 2004) that the AMP-activated protein kinase (AMPK) might contribute to hypoxia-response coupling, in the context of hypoxic pulmonary vasoconstriction (HPV). This proposal was founded on the assertion that mitochondria of O₂-sensing cells are uniquely responsive to hypoxia due to their selective expression of a specialised form of cytochrome C oxidase (COX), the kinetics of which were predicted to be exquisitely sensitive to changing *PO*₂ within the physiological range (Mills and Jobsis, 1970, 1972). Importantly, in developing my hypothesis I also accounted for the findings of Leach and co-workers, who identified little or no evidence of a fall in cellular ATP within pulmonary arterial myocytes using P-NMR spectroscopy (Leach et al., 2000). Accepting the mitochondrial hypothesis on O₂-sensing, the only obvious explanation for there being no fall in ATP availability during hypoxia was that any initial fall in ATP supply and associated ADP accumulation would be immediately compensated for by the adenylate kinase reaction, leading to consequent increases in the AMP/ATP ratio (Dzeja and Terzic, 2003; Panayiotou et al., 2014). Coincidentally there was a growing understanding of the significance of the AMP-activated protein kinase (AMPK) to cell-autonomous metabolic homeostasis, studies having revealed that AMPK is coupled to mitochondrial metabolism through its exquisite sensitivity to changes in cellular AMP/ATP and ADP/ATP ratios, and acts to maintain ATP supply in the longer term (Hardie et al., 1998). It was immediately apparent that, being a serine threonine kinase, AMPK might also phosphorylate targets outside of those canonical pathways by which it regulates cell-autonomous metabolic homeostasis. At this time the only way to test my hypothesis was through pharmacological intervention, by activating AMPK directly using AICAR (Corton et al., 1995) and indirectly through mitochondrial inhibition using the biguanide phenformin (Hawley et al., 2003). Our preliminary investigations were encouraging, AICAR inducing constriction of pulmonary arteries and mimicking precisely the effects of hypoxia in this respect. I then elaborated on my initial hypothesis (Evans, 2006a, b), proposing that, through natural selection, the role of AMPK in regulating metabolic homeostasis might have been extended to incorporate system-level control of ventilatory responses during hypoxia, and thus oxygen and energy (ATP) supply to the whole body. This raised the possibility that AMPK deficiency or excess might contribute to hypoxic pulmonary hypertension and sleep apnoea (Evans, 2006b). The aim of the present article is to review recent progress.

The AMP-activated protein kinase

AMPK is a cellular energy sensor that acts to maintain energy homeostasis (Figure 1). It exists as heterotrimers comprising one of two catalytic α subunits, in combination with one each of two β and three γ regulatory subunits, which together may form at least 12 different heterotrimeric subunit combinations (Hardie, 2014a; Hardie, 2014c). Evidence is now emerging in support of the view that different subunit combinations may be selected by a given cell type, that each combination may exhibit different sensitivities to activation by AMP and ADP and thus metabolic stresses, and that each may selectively phosphorylate and regulate a different spectrum of target proteins (Ross et al., 2016b). These possibilities formed the central pillars of my hypothesis regarding system-level control of whole-body metabolic homeostasis through AMPK (Evans, 2006a).

There are four nucleotide binding sites (*CBS repeats*) on the γ subunit, of which only sites designated 1, 3 and 4 may ever be occupied (Ross et al., 2016b). Binding of AMP to the γ subunit causes a 10-fold increase in AMPK activity by allosteric activation, with further activation of up to 100-fold generated by binding of either AMP or ADP through their promotion of phosphorylation and inhibition of dephosphorylation at Thr172 on the α subunit (Figure 1). Each of these effects is opposed by ATP (Gowans et al., 2013; Ross et al., 2015). Thr172 is primarily phosphorylated by the tumour suppressor kinase LKB1, which appears to be constitutively active but phosphorylates AMPK more rapidly when AMP and/or ADP is bound to the γ subunit, due to reduced dephosphorylation at Thr172 (Hawley et al., 2003; Ross et al., 2016a; Sakamoto et al., 2004). There is also an alternative Ca^{2+} -dependent activation mechanism, mediated through the calmodulin-dependent protein kinase CaMKK2 (Hawley et al., 2005; Woods et al., 2005), which phosphorylates Thr172 and thus activates AMPK in an AMP-independent manner (Hardie, 2007; Hardie, 2014a; Hardie, 2014c, d). Contrary to previous proposals (Emerling et al., 2009), however, there is little evidence to support the view that AMPK is directly activated by reactive oxygen species (ROS), although ROS may activate AMPK indirectly by inhibiting mitochondrial oxidative phosphorylation and increasing AMP/ATP and ADP/ATP ratios (Auciello et al., 2014; Hawley et al., 2010a). Once activated the classical action of AMPK is to phosphorylate targets that switch off non-essential anabolic processes that consume ATP and switch on catabolic pathways that generate ATP (Hardie, 2007), thereby compensating for

deficits in ATP supply via, for example, reductions in mitochondrial oxidative-phosphorylation.

Intriguingly, in the context of the present discussion, evidence suggested that the genes encoding the α and γ subunits of the AMPK ortholog of yeast *Saccharomyces cerevisiae* (*SNF1* and *SNF4*) support colony level metabolic adaptations (Celenza and Carlson, 1986; Celenza et al., 1989; Mitchelhill et al., 1994; Woods et al., 1994). For example, in a high glucose environment yeast initially grow rapidly using glycolytic metabolism to generate ATP, but when glucose runs low the growth rate slows as yeast undergo diauxic shift towards greater reliance on mitochondrial oxidative-phosphorylation. This adaptation to deficits in substrate supply can be blocked in yeast with *snf1* or *snf4* mutations (Celenza and Carlson, 1986; Celenza et al., 1989; Haurie et al., 2003), rendering these mutants incapable of growing without a source of glucose. In an evolutionary context, this observation raised the possibility that natural selection may have extended the role of AMPK from cell-autonomous metabolic homeostasis to system level adaptations employed by animals to accommodate deficits in O₂ and thus energy supply to the whole body.

Furthermore, as noted above, the fact that AMPK is a serine threonine kinase suggested the capacity for regulation of processes outside of metabolism such as ion channel activity (Figure 1), which our findings (Evans et al., 2005c; Ikematsu et al., 2011; Ross et al., 2011) and those of others have since confirmed. For example, AMPK may phosphorylate and “inactivate” the pore forming α subunit of multiple Ca²⁺-activated potassium channels (K_{Ca}1.1 and K_{Ca}3.1) (Klein et al., 2009; Ross et al., 2011), the voltage-gated potassium channel Kv1.5 (Andersen et al., 2015; Mia et al., 2012; Moral-Sanz et al., 2016) and the ATP-inhibited K_{ATP} channel (Kir6.2) (Chang et al., 2009), or may phosphorylate and “activate” Kv2.1 α subunits (Ikematsu et al., 2011). Thereby AMPK has the potential to increase or decrease cell excitability, in a manner determined by both the cell-specific expression of its subunits and members of the ion channel superfamily, providing the capacity for system-level adjustments to whole-body metabolic status (Evans, 2006a). Expanding on this, evidence is emerging that AMPK may also directly phosphorylate and regulate enzymes key to transmitter biosynthesis (Zhang et al., 2018), receptors (Ahmadi and Roy, 2016), pumps and transporters (Schneider et al., 2015).

AMPK and hypoxic pulmonary vasoconstriction

HPV is initiated by airway hypoxia rather than by vascular hypoxaemia (Bergofsky et al., 1968), through the constriction of pre-capillary resistance arteries coordinated by signalling pathways that are intrinsic to their smooth muscle and endothelial cells (Dipp et al., 2001; Evans et al., 2005a; Gaine et al., 1998; Robertson et al., 2001), and in a manner independent of blood-borne mediators or the autonomic nervous system (Naeije et al., 1989; Robin et al., 1987). The initiation phase of acute HPV is primarily driven by smooth muscle constriction (Dipp et al., 2001), with a threshold $PO_2 \approx 80$ mmHg (Dipp and Evans, 2001), after which the magnitude of HPV increases with the degree of hypoxia. It is now generally accepted that HPV relies on the modulation of mitochondrial metabolism by hypoxia, the overriding question has been how?

Recent investigations have demonstrated that NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2 (NDUFA4L2) (Tello et al., 2011) and cytochrome C oxidase subunit 4 isoform 2 (COX4I2) (Fukuda et al., 2007; Huttemann et al., 2001), two nuclear encoded atypical subunits of the mitochondrial electron transport chain, are constitutively expressed under normoxia not only by pulmonary arterial myocytes (Aras et al., 2013; Huttemann et al., 2012) but also by oxygen-sensing carotid body type I cells (Zhou et al., 2016). By contrast, in a variety of other cell types NDUFA4L2 and COX4I2 expression is ordinarily low, but may be increased during prolonged hypoxia (Fukuda et al., 2007; Huttemann et al., 2001). This led to the proposal that constitutive expression of NDUFA4L2 and COX4I2 in O_2 -sensing cells might determine the affinity of their mitochondria for O_2 and thus confer, in part, the capacity of these cells to monitor changes in arterial O_2 supply. Accordingly it has been shown that HPV is occluded by deletion of the gene encoding mitochondrial protein COX4I2 (Sommer et al., 2017), confirming the predictions of Mills and Jobsis regarding not only the site but also the mechanism by which hypoxia impacts O_2 -sensing cells (Mills and Jobsis, 1970, 1972). This is evident from the fact that allosteric modulation of COX is delivered by COX4 in a subtype-specific manner, COX4I1 but not COX4I2 conferring COX inhibition by ATP (Horvat et al., 2006; Huttemann et al., 2001). Consequently, in pulmonary arterial myocytes and carotid body type I cells the rate of O_2 consumption and thus ATP supply via mitochondrial oxidative phosphorylation will not increase during hypoxia as ATP levels fall (Aras et al., 2013; Fukuda et al., 2007; Horvat et al., 2006; Kocha et al., 2015), promoting the adenylate kinase reaction and consequent increases in the AMP/ATP ratio (Dzeja and Terzic, 2003; Panayiotou et al.,

2014). It is also notable that expression of both NDUFA4L2 and COX4I2 may act to limit mitochondrial ROS production during hypoxia, by reducing the activity of complex I and COX, respectively (Fukuda et al., 2007; Huttemann et al., 2001). This is significant because it is evident that COX4I2 deletion (Sommer et al., 2017) may attenuate HPV by blocking hypoxia-evoked increases in mitochondrial ROS and/or cytoplasmic AMP/ATP ratios. All things considered, perhaps the latter of the two is the most likely to be compromised. This is a critical point because it has been argued that ROS may activate AMPK directly through an AMP- and LKB1-independent mechanism (Emerling et al., 2009), although it is most likely that ROS activate AMPK indirectly by inhibiting mitochondrial oxidative-phosphorylation (Auciello et al., 2014; Hawley et al., 2010a).

Consistent with a role for mitochondria in HPV, all mitochondrial inhibitors tested thus far mimic the effects of hypoxia on pulmonary arterial smooth muscle cells at the level of the “oxygen-sensitive” delayed rectifier potassium (K_v) current (Firth et al., 2008; Post et al., 1992). Curiously, however, studies on perfused lungs and pulmonary artery rings have shown that only some mitochondrial inhibitors constrict pulmonary arteries and occlude further constriction during hypoxia, while others block HPV without first inducing constriction (Leach et al., 2001; Weissmann et al., 2003). In this respect it is important to note that HPV fails under near anoxic conditions ($< 1\%$ oxygen), i.e., there is a PO_2 window within which pulmonary artery constriction may be initiated by hypoxia. It is therefore notable, for example, that the NAD(P)H/NAD(P)⁺ ratio in dorsal root ganglion neurons, which do not serve to monitor oxygen supply, exhibits no shift until the PO_2 falls to near anoxic levels (~ 5 mmHg); i.e., the PO_2 at which HPV begins to fail (Dipp et al., 2003). Why might this be significant? Strictly speaking, it is the “anoxic” and not the “hypoxic” condition that mitochondrial inhibitors would mimic at concentrations that ablate oxidative-phosphorylation. Therefore, an explanation for the inconsistency of outcome with respect to the effects of mitochondrial inhibitors on HPV and the pO_2 window within which HPV is triggered, may ultimately be provided by a greater understanding of the impact on pulmonary vascular function of degrees of metabolic stress. After all, opposing pulmonary vasoconstriction during anoxia might well serve to maximise gaseous exchange within the lungs in extremis.

These considerations bring us back nicely to AMPK, which is activated by all mitochondrial inhibitors and thus hypoxia, in a manner dependent on the degree of inhibition of mitochondrial oxidative-phosphorylation (Hawley et al., 2010b). It is quite

possible, however, that during more extreme metabolic stress, such as anoxia, AMPK may play its now classical role and “switch off” non-essential ATP-consuming processes in order to ensure cell survival, and may not under these conditions function itself to drive constriction. Alternatively, there may simply be insufficient ATP availability during anoxia to allow for continued phosphorylation and activation of AMPK.

What of the evidence supporting a role for AMPK in HPV (Figure 1)? Our initial studies (Evans et al., 2005a) showed that exposure of pulmonary arterial smooth muscle to hypoxia (15-20 mmHg) precipitates an increase in the cellular AMP/ATP and ADP/ATP ratios, concomitant activation of AMPK and phosphorylation of acetyl-CoA carboxylase (ACC; an established marker for AMPK action), despite the fact that cellular ATP levels remain remarkably stable in the presence of hypoxia (Leach et al., 2000). Moreover, inhibition of mitochondrial oxidative phosphorylation by phenformin (Owen et al., 2000) evoked increases in NAD(P)H autofluorescence, AMPK activation and ACC phosphorylation in pulmonary arterial smooth muscle cells (Evans et al., 2005b). AMPK activation and ACC phosphorylation were also induced by AICAR, which activates AMPK directly rather than by inhibiting the mitochondrial electron transport chain, through uptake into cells and subsequent metabolism to the AMP mimetic, ZMP (AICAR monophosphate; (Corton et al., 1995). Regardless of their respective mechanism of action, phenformin and AICAR induced an increase in the intracellular Ca^{2+} concentration in acutely isolated pulmonary arterial smooth muscle cells and did so by mobilising sarcoplasmic reticulum (SR) stores via ryanodine receptors, as does hypoxia. Most significantly, AMPK activation by AICAR evoked a slow, sustained and reversible constriction of pulmonary artery rings; an action not mimicked by phenformin due to confounding effects on smooth muscle function (Evans, unpublished observation). Moreover, the sustained phase of HPV and pulmonary artery constriction in response to AICAR exhibited strikingly similar characteristics, namely: (1) A requirement for smooth muscle SR Ca^{2+} release via ryanodine receptors that is retained, if attenuated, after removal of extracellular Ca^{2+} ; (2) Ca^{2+} influx into and vasoconstrictor release from the pulmonary artery endothelium (Evans et al., 2005b). Consistent with these findings, HPV was also inhibited by the non-selective AMPK antagonist Compound C (Robertson et al., 2008), although any action of this agent should be considered with caution because in a screen of 70 protein kinases Compound C was shown to inhibit at least ten other kinases more potently than

AMPK (Bain et al., 2007).

Further significant support for our original proposals has now been gathered by use of more selective small molecule activators of AMPK, A769662 (Goransson et al., 2007; Rajamohan et al., 2015) and Compound 13 (Hunter et al., 2014)), and activated (thiophosphorylated) recombinant human AMPK heterotrimers (Ross et al., 2011). Intracellular dialysis of activated human AMPK or extracellular application of AMPK activators resulted in phosphorylation and inhibition of recombinant Kv1.5 channels expressed in HEK293 cells, the archetypal O₂-sensing K⁺ channel expressed by pulmonary arterial myocytes (Post et al., 1992). Most significantly, A769662 and Compound 13 not only inhibited Kv currents in acutely isolated pulmonary arterial smooth muscle cells, but occluded further inhibition of these K⁺ currents by hypoxia and mitochondrial poisons (Moral-Sanz et al., 2016). Therefore, AMPK activation mimics the effects of hypoxia on pulmonary arterial smooth muscle cells at the molecular, cellular and system level.

This leaves us with perhaps the most important question – is the LKB1-AMPK signalling cascade necessary for HPV? Our preliminary studies on mice in which either LKB1 (*Stk11*), CaMKK2 (*Camkk2*), AMPK- α 1 (*Prkaa1*) or AMPK- α 2 (*Prkaa2*) have been deleted or knocked down are entirely consistent with this view (Moral-Sanz et al., 2015; (Moral-Sanz et al., 2018).

AMPK and the HVR

As mentioned above, a natural extension of my hypothesis on the role of AMPK in reflex responses to hypoxia was to incorporate the HVR, and explore whether AMPK might be of general importance to system-level coordination of O₂ supply. At the time the general consensus was that the carotid bodies, the primary peripheral arterial chemoreceptors of mammals, govern the entire ventilatory response to falls in arterial PO₂ (Prabhakar, 2000). Our preliminary investigations into the role of the LKB1-AMPK signalling pathway appeared entirely consistent with this view, in that pharmacological studies suggested that the AMPK agonist AICAR (Corton et al., 1995) activated carotid body type I cells and increased afferent discharge from isolated carotid bodies (Evans et al., 2005b; Wyatt et al., 2007). These actions were also inhibited by the AMPK antagonist Compound C (Wyatt et al., 2007). Consistent with this, conditional deletion of the gene encoding LKB1 virtually abolished the capacity for

carotid body afferent fibre discharge during hypoxia and attenuated the HVR (Evans, 2012; Mahmoud et al., 2015a). Contrary to these findings and against our expectations, however, AMPK- α 1/2 deletion failed to attenuate afferent discharge from the carotid body, yet conferred even greater attenuation of the HVR (Mahmoud et al., 2015b) than LKB1 deletion (unpublished).

How did the contraindications provided by our pharmacological studies arise? As mentioned previously, Compound C is a very non-selective kinase inhibitor, which in a screen of 70 protein kinases was shown to inhibit at least 10 other kinases more potently than AMPK (Bain et al., 2007). Moreover off target effects of other pharmacological tools have also been identified, such as inhibition by AICAR of adenosine transporters (Gadalla et al., 2004), which would raise extracellular adenosine concentrations, activate type I cell adenosine receptors and thus increase afferent discharge from the carotid body (Murali and Nurse, 2015). We attempted to cover for this possibility by completing some experiments using AICAR in the presence of the adenosine receptor antagonist 8-P-(sulphophenyl)-theophylline, but this strategy may have been less successful than we presumed at the time (Wyatt et al., 2007). Alternatively, AICAR-mediated reductions in the adenylate pool and ATP (Hasenour et al., 2014; Lantier et al., 2014) could similarly impact type I cell activities. Whatever the explanation, it is clear that AMPK is not *necessary* for type I cell activation during hypoxia. Accordingly, recent studies on the actions of two different AMPK activators, AICAR and A769662, suggest that these agents neither precisely mimic the effects of hypoxia on, nor induce pronounced activation of carotid body type I cells (Buckler, 2015; Kim et al., 2014), and our own further investigation now supports this view (Evans, unpublished).

Nevertheless, we have inadvertently uncovered a split in the dependency on LKB1 and AMPK, respectively, of carotid body activation during hypoxia on the one hand and the HVR on the other. The reasons for this remain to be resolved, but experimental outcomes perhaps point to hierarchical control of the respiratory network by LKB1, AMPK and perhaps one or more of the 12 AMPK-related kinases; unlike AMPK, the activities of AMPK-related kinases are in general regulated through their degree of expression and constitutive phosphorylation by LKB1, which occurs independent of cellular metabolic status, but may in some cases impact cell-autonomous metabolic homeostasis (Lizcano et al., 2004). Given that afferent discharge is, in great part,

triggered by exocytotic release of ATP from type I cells (Murali and Nurse, 2016), it is quite plausible that LKB1 may maintain, in an AMPK-independent manner, the capacity for ATP synthesis and / or exocytosis within type I cells, and thus afferent discharge from the carotid body. In keeping with this, studies on other cell types suggest that LKB1 may govern glucose homeostasis (Koh et al., 2006; Shaw et al., 2005) and mitochondrial function (Gan et al., 2010; Gurumurthy et al., 2010) independent of AMPK, either directly or through constitutive phosphorylation of an AMPK-related kinase (Choi et al., 2015; Lizcano et al., 2004; Patel et al., 2014). Supporting this, LKB1 deletion has been shown to decrease mitochondrial membrane potential and basal ATP levels in certain cell types (Gan et al., 2010; Gurumurthy et al., 2010; Swisa et al., 2015). Likewise, carotid body type I cells lacking LKB1 may be unable to sustain appropriate cellular energy charge and activity due to defective mitochondrial function, either at rest or during exposure to metabolic stresses such as hypoxia.

This takes us back to ATP, ADP and AMP levels and the inhibition during hypoxia of type I cell K^+ channels, which ultimately triggers exocytosis (Delpiano and Hescheler, 1989; Lopez-Barneo et al., 1988; Stea and Nurse, 1991). The principle players in this respect are the large conductance voltage- and Ca^{2+} -activated K^+ current (BK_{Ca}) (Hescheler et al., 1989; Peers, 1990) and the voltage-independent TASK-like leak K^+ current (Buckler, 1997; Kim et al., 2009; Ortega-Saenz et al., 2010); although it should be noted that variations in channel expression may confer identified species differences (Lopez-Lopez et al., 1993; Perez-Garcia et al., 2004) and contribute to changes of O_2 sensitivity during postnatal maturation (Hatton et al., 1997; Wasicko et al., 2006). It is now clear that hypoxia (and hypercapnia) principally acts to depolarise type I cells by inhibiting TASK1/3 K^+ channels (Buckler, 2015), leading to Ca^{2+} entry through voltage-gated Ca^{2+} channels, consequent exocytosis and ATP release. Moreover, in the absence of a determining role for AMPK (Kim et al., 2014; Mahmoud et al., 2015b), evidence now supports the view that TASK K^+ channels directly monitor the adenylate pool (Varas et al., 2007), closing when ATP levels fall consequent to the inhibition by hypoxia of mitochondrial oxidative phosphorylation (Wyatt and Buckler, 2004). AMPK does however phosphorylate and, like hypoxia, inhibit BK_{Ca} channels of carotid body type I cells (Ross et al., 2011), the archetypal O_2 -sensing K^+ channel (Lopez-Barneo et al., 1988; Peers, 1990). This action will clearly have functional consequences with respect to transmitter release, conceivably by modulating action potential firing patterns (Duncan et al., 2015), which may impact Ca^{2+} influx under certain conditions

(Evans et al., 2005b; Kim et al., 2014). However, these matters remain to be resolved. Nevertheless, it is clear from our own findings that all pathways key to carotid body type I cell activation during hypoxia must be, in some way, dependent on the continued expression of LKB1, but not AMPK, and a sufficiency of mitochondrial function and / or ATP supply. So how can both LKB1 and AMPK deletion block the HVR, when deletion of the latter does not adversely affect carotid body activation during hypoxia (Evans, 2012; Mahmoud and Evans, 2012; Mahmoud et al., 2015b)?

It is quite plausible that peripheral chemosensors are not the sole arbiters of the HVR, as has been suggested by investigations on the evolution of ventilatory control systems, most notably with respect to the demonstration that O₂-sensing occurs and a component of the HVR arises at the level of the caudal brainstem in amphibians, with both the location and influence of the primary peripheral chemosensors changing during the ascent from gill breathing tadpole to lung-assisted, air breathing adult (Jia and Burggren, 1997; Porteus et al., 2011). In fact one could reasonably argue that evolutionary pressures have periodically led to the reconfiguration of peripheral chemoreceptor inputs (Porteus et al., 2011) about a common ancestral hypoxia-sensor within the caudal brainstem, that underpins signal integration and thus acts as the “gatekeeper” of respiratory adjustments during hypoxia.

While the aforementioned findings run counter to the view that increased afferent discharge from carotid body to brainstem alone determines the ventilatory response to a fall in arterial *PO*₂, they do provide substantial support for an alternative yet inclusive perspective, namely that the HVR is determined by the coordinated action of the carotid body and an hypoxia-responsive circuit within the brainstem (Curran et al., 2000; Evans et al., 2016; Gourine and Funk, 2017; Mahmoud et al., 2015b; Smith et al., 1993; Teppema and Dahan, 2010). To date little emphasis has been placed on the role of hypoxia-sensing at the level of the brainstem, perhaps because the HVR is so effectively abolished by resection of the carotid sinus nerve in humans (Wade et al., 1970). Yet brainstem hypoxia induces an HVR when in receipt of normoxic carotid body afferent inputs (Curran et al., 2000; Smith et al., 2010), and directly activates (Roux et al., 2000) subsets of neurons within the nucleus tractus solitarius (NTS) (Pascual et al., 2002) and rostral ventrolateral medulla (Nolan and Waldrop, 1993; Sun et al., 1992; Sun and Reis, 1993; Teppema and Dahan, 2010). This may in turn be supported by direct activation during hypoxia of lactate and / or ATP release from astrocytes (Angelova et al., 2015; Gourine and Funk, 2017; Magistretti and Allaman, 2018; Marina et al., 2013).

Moreover and consistent with the fact that our gene deletion strategy targeted catecholaminergic neurons (ectopic expression aside), extensive investigations have demonstrated that following carotid body resection, hypoxia-responsive catecholaminergic neurons of the caudal brainstem may underpin partial recovery of the HVR in a variety of animal models (Roux et al., 2000; Roux and Villard, 2010; Smith and Mills, 1980). Accordingly, dysfunction of these neurons has been shown to underpin hypoventilation and apnoea associated with Rett syndrome, which is exacerbated during hypoxia (Roux and Villard, 2010). Adding to this, it is evident that COX4I2 may, as for carotid body type I cells and pulmonary arterial myocytes, be expressed under normoxia by certain CNS neurons (Horvat et al., 2006), perhaps rendering mitochondrial oxidative phosphorylation within such cells exquisitely sensitive to falls in local PO_2 within the physiological range. AMPK activation in a specialised subset of brainstem catecholaminergic neurons could thus be triggered during hypoxia, supporting the delivery of increased respiratory drive required to protect against hypoventilation and apnoea. Evidence of this was provided by examination of brainstem function in AMPK- $\alpha 1/\alpha 2$ knockout mice by functional magnetic resonance imaging (fMRI), which identified reductions in oxygen consumption during hypoxia (indicative of reduced activation) within discrete dorsal and ventral nuclei of the caudal brainstem, despite the fact that carotid body afferent input responses were retained (Mahmoud et al., 2015b). This has been corroborated by analysis of immediate early gene (*cfos*) expression. The caudal location relative to Bregma of the dorsal active region is consistent with areas of the NTS (Figure 2) that are activated during hypoxia and which represent the primary site of receipt of carotid body afferent input (Guyenet, 2000; Hirooka et al., 1997; King et al., 2012; Koshiya and Guyenet, 1996; Teppema and Dahan, 2010). Here AMPK- $\alpha 1/\alpha 2$ deletion may attenuate the activation during hypoxia of C2 adrenergic and/or A2 nor-adrenergic neurons proximal to the midline and the area postrema (Mahmoud et al., 2015b). Significantly, A2 neurons of the area postrema / NTS provide afferent inputs to and determine, together with the carotid body, activation by hypoxia of A1/C1 neurons within the ventrolateral medulla (Alheid et al., 2011; Hirooka et al., 1997), the position of which (Alheid et al., 2011) aligns well with the ventral active region identified by fMRI analysis (Mahmoud et al., 2015b); by contrast projections of the NTS mostly avoid key components of the rCPGS (Alheid et al., 2011), namely the Böttinger and

pre-Bötzing complexes (Smith et al., 1991).

Our findings therefore suggest that the HVR is attenuated by loss of AMPK function at the level of the caudal brainstem, within a neuronal circuit spanning the C2/A2 neurons of the NTS and A1 neurons of the ventrolateral medulla. This is consistent with optogenetic and pharmacological interventions at the level of the NTS (King et al., 2012; Yamamoto et al., 2015), and the proposal that NTS neurons lie on the sensory side of the central respiratory network (Aicher et al., 1996; Vardhan et al., 1993). Surprisingly, we observed pronounced right-left asymmetry of brainstem activation during hypoxia, which may provide for specialisation sufficient to prevent delays in respiratory responses to hypoxic stress by limiting conflicting outputs from each side of the brain (Vallortigara et al., 1999), as has been proposed previously with respect to cognitive performance (Dadda et al., 2009). Further investigation will be required to determine how right-left asymmetry may be orchestrated by the complex interplay of neurotransmitters deployed during hypoxia and the role of AMPK in such processes of selection. In this respect it is notable that both C2 and A2 neurons are both catecholaminergic and glutamatergic (Stornetta et al., 2002; Vardhan et al., 1993), and that 6-10% of TH-positive C2, A2 and A1 neurons also express neuronal nitric oxide synthase, which supports the HVR by synthesizing NO (Gozal et al., 1997) and/or S-nitrosothiols (Lipton et al., 2001), and in a manner that may be facilitated by AMPK (Murphy et al., 2009).

It could be argued that AMPK- $\alpha 1/\alpha 2$ deletion in catecholaminergic cells simply leads to the failure of central integration and transduction of peripheral chemoafferent inputs and consequent failure of the HVR, due to the inability of affected neurons to maintain appropriate levels of activity when exposed to metabolic stress (Culmsee et al., 2001). However, following AMPK- $\alpha 1/\alpha 2$ deletion carotid body afferent discharge remained exquisitely sensitive to falls in PO_2 and ventilatory responses to hypercapnia remained unaffected even during severe (8%) hypoxia. Given that AMPK- $\alpha 1/\alpha 2$ deletion would have occurred in all catecholaminergic neurons irrespective of location and whether or not they are O_2 - or CO_2 -sensitive, these findings indicate that AMPK- $\alpha 1/\alpha 2$ deficiency does not block the HVR by compromising, *per se*, the capacity during hypoxia for activation of chemosensory catecholaminergic neurons, exocytosis nor effective delivery of increased respiratory drive. Rather, outcomes suggest that AMPK- $\alpha 1/\alpha 2$ deficiency may in some other way selectively interfere with the integration and onward

relay of afferent input responses at the level of the NTS. This is consistent with the observation that neuronal integrity during hypoxia may be preserved, in part, by AMPK-independent mechanisms (Cheng et al., 2011) that maintain ATP supply by accelerating glycolysis in a manner supported by mobilisation of astrocyte glycogen stores (Almeida et al., 2004) and lactate release (Magistretti and Allaman, 2018). AMPK must therefore support the modulation by hypoxia of discrete nuclei within the caudal brainstem that deliver increased drive to breathe via neural networks that modulate the respiratory central pattern generators (rCPGs) (Guyenet, 2014), and which may also coordinate functional hyperaemia (Bucher et al., 2014). Whatever the mechanism it is most likely neurogenic and highly localised, given that AMPK deficiency in smooth muscles does not affect the HVR or systemic arterial blood pressure regulation during hypoxia, while the latter but not the former remains unaltered following AMPK deletion in catecholaminergic neurons (MacMillan and Evans, 2018). Given that AMPK- $\alpha 1/\alpha 2$ deficiency in catecholaminergic neurons precipitates apnoea during hypoxia and respiratory failure under anaesthesia (Mahmoud et al., 2016), we cannot entirely rule out the possibility that suppression of the HVR may be allied to exacerbation of the “Cushing reflex” (Guyenet, 2000; Paton et al., 2009); classically, the Cushing reflex refers to the induction of systemic hypertension, bradycardia and apnoea consequent to intracranial hypertension. However, the Cushing reflex is only elicited under anaesthesia and by ischemic hypoxia ($\sim 1\% \text{ O}_2$), and is maintained or enhanced by hypercapnia (Guyenet, 2000; Harris et al., 1998; Paton et al., 2009). By contrast, hypoxic ventilatory depression was evident in conscious AMPK- $\alpha 1/\alpha 2$ knockouts during mild or severe hypoxia, as were deficits in brainstem activity, and this was reversed rather than exacerbated by hypercapnia. If this AMPK-dependent, hypoxia-responsive nucleus does indeed exist, why has it not been located before? Perhaps we are dealing with an interdependent circuit mechanism, with multiple points of signal integration. In this context and in light of all things above, we need now consider why:

1. The degree of block by AMPK- $\alpha 1/\alpha 2$ deletion of the HVR is increased in a manner directly related to the severity of hypoxia (Mahmoud et al., 2015b);
2. The HVR can be triggered by CNS hypoxia alone, providing there is continued receipt of basal (normoxic) afferent input from the carotid bodies (Smith et al., 2010);

3. The HVR may be blocked by interference at any point within this circuit, e.g., by carotid body resection (Wade et al., 1970) or AMPK- $\alpha 1/\alpha 2$ deletion (Mahmoud et al., 2015b).

To account for these points, we proposed that LKB1-AMPK signalling pathways support coincidence detection and thus signal integration at either a single node or multiple nodes within and thus activation of a hypoxia-responsive circuit that encompasses, at the very least, C2/A2 neurons within the NTS and ventrolateral A1 neurons (Evans et al., 2016), due to the capacity for: (1) AMPK activation by increases in AMP/ATP and ADP/ATP ratios, and LKB1 (Hardie, 2014b) determined by “local hypoxic stress” (decreased ATP supply); (2) Augmentation of this response by “applied metabolic stress” (increased ATP usage) due to the further activation of these neurons through afferent inputs from peripheral chemoreceptors to the NTS (Figure 2). In turn this may impact ventrolateral A1 neurons and perhaps additional downstream aspects of the cardiorespiratory network. Afferent input and brainstem hypoxia could thereby determine, each in part, the set-point about which AMPK and thus the brainstem respiratory networks are activated during hypoxia. To test this hypothesis further, we must first identify the hypoxia-responsive nuclei within the brainstem and in doing so determine whether this nucleus incorporates adrenergic (C2) and/or nor-adrenergic (A2) neurons. Thereafter we may examine the relative contributions made by AMPK-dependent modulation of cellular metabolism (Hardie, 2014b), ion channels and thus neuronal firing frequency (Ikematsu et al., 2011), and transmitter release (Lipton et al., 2001; Murphy et al., 2009) to efferent outputs that deliver increased drive to breathe. Whether we consider HPV or the HVR, it would appear that it is AMPK- $\alpha 1$ but not AMPK- $\alpha 2$ that is critical to reflex responses during hypoxia (see for example (Mahmoud et al., 2016)), although AMPK- $\alpha 2$ may play a supporting role during severe hypoxia. This is significant and brings us back to evolution, because investigations of others on high-altitude, *Andean* populations suggest that the gene encoding AMPK- $\alpha 1$ (*PRKAA1*) has been influenced by natural selection through single nucleotide polymorphisms (Bigham et al., 2014).

AMPK sleep apnoea and pulmonary hypertension

The significance of evolutionary action on AMPK- $\alpha 1$ may lie in the fact that pulmonary hypertension (Lahm et al., 2014) and sleep disordered breathing (Chau et al., 2012) are

associated with ascent to altitude (Ainslie et al., 2013). In short, it may be beneficial to insure against aberrant expression of AMPK- α 1.

This is evident from our finding that AMPK- α 1 deficiency in catecholaminergic cells precipitates late hypoxic ventilatory depression culminating in apnoea rather than hyperventilation, reminiscent of the HVR of neonates (Gozal, 2016). Furthermore, hypoxic ventilatory depression due to AMPK deficiency in catecholaminergic neurons is enhanced under anaesthesia, ultimately leading to respiratory failure (Mahmoud et al., 2016). Thus AMPK- α 1 deficiency is strongly indicated in the pathogenesis of sleep apnoea syndrome, where management of anaesthesia is also critical (Boushra, 1996). In the context of pulmonary hypertension, however, we meet a paradox, because while AMPK- α 1 may drive HPV and thus acute hypoxic pulmonary hypertension, recent investigations of our own (unpublished since 2016) and others suggest that AMPK deficiency may promote pulmonary hypertension (Omura et al., 2016; Zhang et al., 2018). Future investigations will determine how AMPK can be both friend and foe.

Summary

A growing body of evidence now supports the proposal that AMPK is key to O₂ and thus energy (ATP) supply to the body as a whole, through its contribution to the governance of ventilation-perfusion matching at the lung and the HVR. Aberrant AMPK expression or activity may therefore compromise system responses to hypoxia, precipitating pulmonary hypertension (Lahm et al., 2014) and sleep disordered breathing (Chau et al., 2012). Notably, pulmonary hypertension and sleep disordered breathing are associated not only with ascent to altitude (Ainslie et al., 2013) but metabolic syndrome-related disorders such as obesity and type 2 diabetes (Chau et al., 2012; Vgontzas et al., 2005), which are in turn associated with cell-specific changes to AMPK expression patterns (Ruderman et al., 2013). Further investigations into the role of AMPK in the regulation of ventilatory and vascular function in health and disease are therefore warranted.

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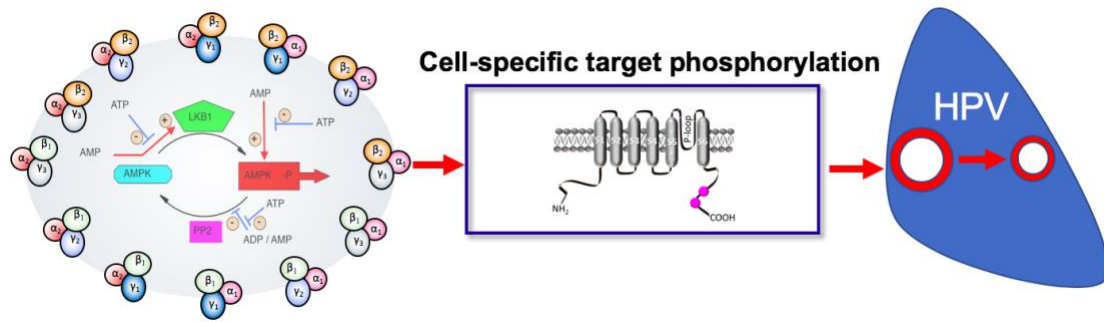


Figure 1. AMPK and hypoxic pulmonary vasoconstriction. Schematic shows the mechanism by which increases in the cellular AMP/ATP and ADP/ATP ratios may activate 12 AMPK heterotrimeric subunit combinations, leading to target phosphorylation and induction of, for example, hypoxic pulmonary vasoconstriction (HPV).

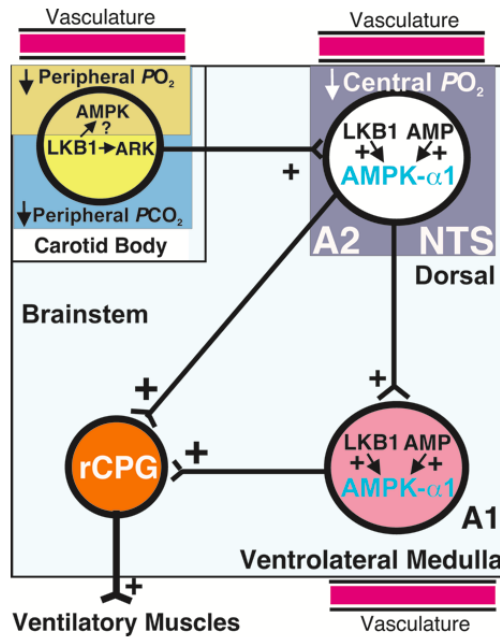


Figure 2. Circuit mechanism by which LKB1 and AMPK may confer hierarchical control of the hypoxia-responsive respiratory network. Schematic presents a minimal model describing the integration of carotid body afferent input responses with local hypoxia by brainstem respiratory networks. NTS = nucleus tractus solitarius.